Temperature-Stable Glycosylated Recombinant Chicken Cystatin And The Use Thereof

Field of the Invention

The present invention relates to the field of glycosylation of protein. Specifically, the present invention relates to glycosylation of chicken cystatin, wherein the chicken cystatin could be produced by genetic engineering technique. The glycosylated recombinant chicken cystatin of the present invention has improved stability to temperature.

Background of the Invention

The cystatin superfamily includes a number of cysteine protease inhibitors that are widely distributed in tissues and body fluids of mammalians (*6*). Chicken cystatin, a well-studied cysteine protease inhibitor, is a small non-glycosylated protein having 116 amino acids linked with two disulphide bonds (*1, 5, 6, 18, 34*), which has been crystallized and subjected to the preliminary X-ray crystallographic studies (*10, 11*). Chicken cystatin is a reverse, tight-binding inhibitor of cysteine proteases such as papain and tissue protease B and L, and is considered to contribute to physiological control in which said proteases participate (*7, 8, 9, 23, 24, 26, 31*). Chicken cystatin inhibits the degradation of proteins in organisms and the softening of muscles of animals when said animals were dead. In food industries, for example, chicken cystatin is added to surimi for the inhibition of endogenous proteolysis of the surimi, thereby reducing the gel softening of said surimi.

However, it was found that chicken cystatin is stable to heat (*18*), but unstable to freezing or freeze-drying (*1, 20*). Application of chicken cystatin on the inhibition of autolysis or endogenous proteolysis was greatly restricted by its lower, unsatisfactory freezing tolerance. Especially, chicken cystatin, when added to surimi, is unstable and easily loses its activity in the freezing-thawing process of surimi. For expanding the use of the chicken cystatin in frozen products, the structure of chicken cystatin may need to be modified for increasing its flexibility and resistance to a freezing-thawing process.

Natural chicken cystatin is not a glycoprotein since there is no N-glycosylation site in its amino acid sequence. There was no teaching or suggestion in the art that change in one or more amino acid residues in the amino acid sequence of chicken cystatin could improve the stability of chicken cystatin to temperature. Further,

there was no teaching or suggestion in the art that glycosylation of the changed amino acid residue(s) in the amino acid sequence of chicken cystatin could improve the stability of chicken cystatin to temperature.

The inventors of the present invention found that change in just an internal amino acid residue of chicken cystatin that is not located in its active site, i.e. Asn_{106}-Ile_{108}→Asn_{106}-Thr_{108}, by employing site-directed mutagenesis and genetic engineering technique without changing or destroying the stereo structure of the active site of chicken cystatin, could facilitate the glycosylation of said Asn_{106}, whereby the stability of chicken cystatin to temperature could be significantly improved. The Asn_{106}-glycosylated, modified chicken cystatin of the present invention, when for example added to surimi, has the desired inhibitory function even if said surimi is treated with several freezing-thawing cycles.

Summary of the Invention

The present invention relates to a N-glycosylation-modified recombinant chicken cystatin, wherein Asn_{106}-Ile_{108} in its amino acid sequence is modified to Asn_{106}-Thr_{108}. The Asn_{106}-glycosylated, modified recombinant chicken cystatin of the present invention, when for example added to surimi, is stable to temperature and has the desired function in the inhibition of thermal degradation of surimi even if said surimi is treated with several freezing-thawing cycles. The surimi is preferably derived from nemipterid, mackerel or cod.

In one aspect, the present invention relates to a nucleic acid molecule encoding the N-glycosylation-modified recombinant chicken cystatin of the present invention, wherein the triplet codon encoding the 108^{th} amino acid in the amino acid sequence of chicken cystatin is changed from AGT to TCA or its degenerate codons.

In another aspect, the present invention relates to an expression vector comprising the nucleic acid molecule encoding the N-glycosylation-modified recombinant chicken cystatin of the present invention. In a preferred embodiment, the expression vector is the expression vector pGAPZαC containing GAP promoter.

In yet another aspect, the present invention relates to a transformant harboring the expression vector. In a preferred embodiment, the host cell to be transformed is yeast. In a more preferred embodiment, the yeast is *Pichia pastoris*. In the most preferred embodiment, the yeast is *Pichia pastoris* strain X-33.

In yet another aspect, the present invention relates to a method for producing the N-glycosylation-modified recombinant chicken cystatin, which comprises the steps of culturing a nutritional medium with the yeast transformant of the present invention for producing the N-glycosylation-modified recombinant chicken cystatin,

and recovering said N-glycosylation-modified recombinant chicken cystatin thus obtained.

In still yet another aspect, the present invention relates to a composition for inhibiting the thermal degradation and gel softening of surimi, comprising the N-glycosylation-modified recombinant chicken cystatin of the present invention and an acceptable expander. The expander may preferably be a compatible protein, starch or a combination thereof.

In still yet another aspect, the present invention relates to a method for inhibiting the thermal degradation and gel softening of surimi by using the composition, comprising adding said composition to surimi. In a preferred embodiment, 0.01 to 0.10 active units, preferably 0.02 to 0.05 active units, of the N-glycosylation-modified recombinant chicken cystatin of the present invention are added to 1 g of surimi.

Brief Description of the Drawings

Figure 1 illustrates SDS-PAGE and substrate SDS-PAGE (activity staining) of the Asn_{106}-glycosylation modified recombinant chicken cystatin using 15% of sodium dodecyl sulfate polyacrylamide electrophoresis, wherein lane *M* represents protein marker, lane A represents purified recombinant chicken cystatin, lanes B and E represent substrate (0.1% casein) SDS-PAGE of non- and Asn_{106}-glycosylated chicken cystatin, lane C represents glycosylated chicken cystatin after Sephacryl S-100 HR chromatography, and lane D represents purified glycosylated chicken cystatin.

Figure 2 illustrates the calibration curve for the determination of the molecular weight of purified Asn_{106}-glycosylation modified recombinant chicken cystatin using FPLC Superose 12 chromatography [o : markers; • : purified Asn_{106}-glycosylation modified recombinant chicken cystatin].

Figure 3 illustrates comparison in the thermal stability of recombinant chicken cystatin and glycosylated recombinant chicken cystatin incubated at 30 to 100 °C for 30 min.

Figure 4 illustrates SDS-PAGE analysis of mackerel surimi proteins, in which various treated-mackerel surimi gels were incubated at 50 °C for 90 min. and then at 95 °C for 10 min. and the dissolved proteins were electrophorectically running on a 10% polyacrylamide of SDS-PAGE, wherein line M represents a protein marker of 10 kDa ladder and MHC represents myosin heavy chain.

Detailed Description of the Invention

The present invention relates to a novel glycosylated recombinant chicken cystatin, which can inhibit the thermal degradation and gel softening of fish meat or surimi. The glycosylated recombinant chicken cystatin of the present invention could be produced by employing site-directed mutagenesis and genetic engineering technique.

The site-directed glycosylation of proteins using yeast expression systems has been a new approach to enhance the molecular stability of recombinant protein produced (*21, 28, 36*). *Pichia pastoris*, a methylotrophic yeast, is an efficient system for the production of recombinant proteins with high expression level (*14, 33*). The GAP promoter gene has been characterized and can express recombinant proteins to high levels in *Pichia pastoris* (*35*).

For examining the effect of N-glycosylation on the freezing stability of recombinant chicken cystatin, the cDNAs coding chicken cystatin and its N-glycosylated mutant (Asn_{106}-Ile_{108}→Asn_{106}-Thr_{108}) were cloned into the pGAPZαC expression vector using the GAP as promoter and Zeocin as resistant agent, which pGAPZαC expression vectors obtained were then transformed into *Pichia pastoris* X-33 host cell. The papain-inhibition properties of the recombinant chicken cystatin and Asn_{106}-glycosylated mutant chicken cystatin were evaluated.

Materials and Methods

Strains: Cloning host: *Escherichia Coli* Top 10F'
Expression host: *Pichia pastoris* strain X-33
Plasmids: Cloning vector: pGEM-T Easy vector
Expression vector: pGAPZαC vector

Screening and amplification of cystatin cDNA from chicken lung mRNA

Total RNA from chicken lung was extracted using Trizol RNA extraction kit (Gibco BRL). The single strain cDNA, produced from RT-PCR, was used as a template. Based on the open reading frame from 128 to 478 for chicken cystatin, the oligonucleotide with 5'-CTCGAGAAAAGAGAGGCTGAAGCTAGCGAGGACCGCTCCCGGCTCCTGGG and 5'-TCTAGATTACTGGCACTTGCTTTCCAGCAGTTT, were used as primers for the PCR reactions. Another antisense-primer, TCTAGATTACTGGCACTTGCTTTCCAGCAGTTTAGTTTGG, in which the ^{106}Asn-Gln-^{108}Ile was replaced by Asn-Gln-Thr (shadowed) to create a glycosylation sequence, was used to substitute the 3'-primer in PCR reaction. Restriction sites at 5' end of the primers for *Xho*I and *Xba*I (underlined) were incorporated to facilitate

subcloning of the product. Amplification was performed using proofreading polymerase (Gibco BRL) by polymerase chain reaction for 35 cycles with 30 sec. denaturation at 94 °C; 30 sec. annealing at 55 °C and 50 sec. extension at 68 °C in a DNA thermal cycler (GeneAmp PCR system 2400, Perkin Elmer, Norwalk, CT).

Construction of chicken cystatin expression pGAPZαC plasmid

The standard techniques of molecular cloning were performed mainly according to Sambrook et al. (*32*). The PCR product was cloned into pGEMT Easy vector (Promega) and then transformed into *E. coli* Top 10 F'. After blue/white selection and midi-preparation, the plasmid was digested with *Xho*I and *Xba*I and then ligated into pGAPZαC vector (Novagen).

Transformation and selection in *Pichia pastoris* X-33 expression host

The pGAPZαC plasmid ligated with correct and/or glyco-modified chicken cystatin in-frame DNA sequence were digested with *Bgl*II in GAP promoter region to linearize the vector and then transformed into *Pichia pastoris* X-33, respectively, by using lithium chloride method (*12*). The colonies were selected by plating the transformants on YPDS agar plates (20 g/L tryptone, 10 g/L yeast extract, 20 g/L dextrose, 182.2 g/L sorbitol and 20 g/L agar) containing 100 μg/mL Zeocin. After at least 20 colonies for each treatment were produced, colonies that were integrated into their chromosome with the pGAPZαC-cystatin DNA and/or pGAPZαC-glyco-modified cystatin DNA and had the best expression quantity, were chosen.

Cultivation of *Pichia pastoris* X-33 expression host and isolation of recombinant cystatin

The chosen *Pichia pastoris* strains were cultivated with 5 mL YPDS broth (20 g/L tryptone, 10 g/L yeast extract, 20 g/L dextrose and 182.2 g/L sorbitol) containing 100 μg/mL Zeocin in a 50 mL flask using a shaking incubator (300 rpm) overnight at 30°C, and then 1 mL of the resulting culture was inoculated into 50 mL of fresh YPD broth (20 g/L tryptone, 10 g/L yeast extract and 20 g/L dextrose) in a 250 mL flask, which was then cultivated at 30°C in a shaking incubator (300 rpm) for 4-5 days. The *Pichia pastoris* cells were excluded by 10 min. centrifugation at 3000 × *g*. The supernatant was collected and subjected to the further purification.

Purification of recombinant chicken cystatin

The recombinant chicken cystatin was purified by 40-60% saturated ammonium sulfate, Sephacryl S-100 HR and Superdex 75 chromatography, while Asn_{106}-glycosylation modified chicken cystatin was purified by Sephacryl S-100

HR, Con A Sepharose and FPLC Superose 12 chromatography.

Deglycosylation

The Asn_{106}-glycosylation modified chicken cystatin was first denatured by heating at 100 °C in the presence of 1% SDS for 10 min. The denatured Asn_{106}-glycosylation modified cystatin (0.1 mg) was then added to N-glycosidase F (5 units; BOEHRINGER MANNHEIM) in 20 mM sodium phosphate buffer (pH 7.2) containing 2% Triton X-100 and 0.2% SDS. The resulting sample was incubated at 37°C overnight.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was performed according to Laemmli (*22*) using a mini-gel system (SE 260 vertical gel unit, Hoefer). The concentration of polyacrylamide gel was 15%. After 30 min incubation with 1% β-mercaptoethanol at 50°C, samples were subjected to SDS-PAGE analysis. The staining and destaining were performed according to the method of Neuhoff et al. (*30*). Ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa), bovine trypsin inhibitor (6.2 kDa), insulin (2.3 kDa) were used as markers.

Substrate SDS-PAGE

The substrate SDS-PAGE was performed according to El-Shamei et al. (*17*). A 12 % of polyacrylamide gel containing 0.1 % w/v casein was employed in this study. About 10 to 20 μg of sample protein was applied onto each well of the gels. After electrophoretical running, the gels were pre-washed with 2.5 % Triton X-100 twice for 30 min. to remove SDS. The resulting gels were incubated with papain (0.01 mg/mL) in a 0.10 M phosphate buffer containing 2.0 mM cysteine and 1.0 mM EDTA (pH 6.0) at 37°C for 60 min. The reaction was stopped by a staining solution (a mixture of 0.01 % Coomassie brilliant blue, 40% methanol and 10% acetic acid). After destaining with 25% ethanol and 10% acetic acid, the visible intense blue bands were the active cystatin zones.

Freezing Stability

For investigating the influence of Asn_{106}-glycosylation on the freezing stability of chicken cystatin, the native, recombinant and its Asn_{106}-glycosylation modified cystatins were frozen at -20°C in distilled water and then thawed at a 24-hour interval for 6 days. During each freezing-thawing process, the remaining activities of each cystatin were determined.

Assay of enzyme inhibitory activity

Papain (EC 3.4.22.2) was used for the inhibition assessment. The inhibitory activity of chicken cystatin was assayed by measuring the remaining papain activity using Z-Phe-Arg-MCA as substrate (*4*). The enzyme, papain, in 0.2 M sodium phosphate buffer (pH 6.0) containing 4 mM cysteine and 2 mM EDTA with or without chicken cystatin were pre-incubated at 37°C for 5 min. The enzyme reaction mixture (0.75 mL) comprised 5 μL enzyme, 0.25 mL 0.4 M sodium phosphate buffer (pH 6.0) containing 8 mM cysteine and 4 mM EDTA, 0.295 mL distilled water and 0.20 mL chicken cystatin. The reaction was started by adding 0.25 mL of 40 μM Z-Phe-Arg-MCA solution and stopped by adding 1.0 mL sodium acetate buffer containing 0.1 M sodium monochloroacetate (pH 4.3). The amount of liberated aminomethylcoumarin was determined by a spectrofluorometer at 350 nm for excitation and at 460 nm for emission. One unit of inhibitory activity was defined as the amount of chicken cystatin that could inhibit one unit of the proteolytic activity of papain, which was defined as the amount of papain that could hydrolyze Z-Phe-Arg-MCA and release 1 μmol aminomethylcoumarin within 1 min. at 37°C.

Kinetic measurements

The inhibitory constants (Ki) were calculated by the method of Dixon (*15*). Fixed amounts of papain (a final concentration of 0.005, 0.01 and 0.02 nM for Asn_{106}-glycosylated, wild type and recombinant chicken cystatin, respectively) was incubated with appropriate amounts of inhibitors in a 0.1 M sodium phosphate buffer (pH 6.0) containing 2 mM cysteine, 1 mM EDTA and 0.1% Brij 35 at 40°C for 3 min. The reaction was started by adding various concentrations of substrate (Z-Phe-Arg-MCA; 2, 4 or 10 μM), and the residual activities were measured (*4*). The inhibition constant (Ki) was calculated from the Dixon plots of 1/v vs. [I].

Protein concentration measurements

Protein concentrations were determined by the dye binding method using bovine serum albumin as the standard.

Results

After transforming *Pichia pastoris* X-33 expression host with the pGAPZαC chicken cystatin plasmid, the expression vector was integrated into genomic DNA due to the existence of GAP promoter sequence. Since the chicken cystatin gene was under control of GAP promoter, high level of the recombinant chicken cystatin

was expressed and secreted into the broth by α-factor preprosequence during shaking cultivation. In both strains (with/without glycosylation modified mutants), the highest level of cystatin activities (about 6.33 units/mg) was observed after 2 days shaking cultivation. No significant difference in total cystatin activity between these 2 strains was obtained after 2 days shaking cultivation. Since no significant increase in cystatin activity was observed during the further cultivation, 2-day cultivation was used in this study.

The Asn_{106}-glycosylation modified *Pichia pastoris* strain, which was integrated into its chromosome with N-Q-^{108}I →N-Q-^{108}T mutant chicken cystatin gene down-strand of GAP promoter in its chromosome, expressed 2 recombinant cystatins (separated by Sephacryl S-100 HR gel filtration chromatography). One was a poly glycosylated protein with 40 degree of polymerization (DP), which amounted to about 50 % of the total recombinant cystatins, while the other one was unglycosylated protein or glycoproteins with very low DP.

Examples

Example 1-Measurement of molecular weight

The non-modified recombinant chicken cystatin was purified according to the previous study (*13*), while the Asn_{106}-glycosylated mutant chicken cystatin was purified to electrophoretical homogeneity by Sephacryl S-100 HR (Figure 1, line C), Con A Sepharose (Figure 1, line D) and Superose 12 chromatography (Figure 1, line E). According to the N-terminal sequences analysis, the sequences of these 2 purified recombinant cystatins (with non- or glycosylation modification) were as predicted. The molecular weight (*M*) of the recombinant chicken cystatin (*13*) and its Asn_{106}-glycosylated mutant were 14 and 20.5 kDa (Figure 2), respectively. Both Asn_{106}-glycosylated mutant chicken cystatin and its deglycosylated form were further confirmed by the substrate SDS-PAGE against papain (Figure 1, lines B and E). As indicated in Figure 1, the Coomassie brilliant blue stained unhydrolyzed casein band indicated the existence of papain inhibitor, which was the recombinant cystatins.

Example 2-Stability of the native, recombinant and Asn_{106}-glycosylated recombinant chicken cystatin against freezing

From the comparison of the residual activity of the native, recombinant and its Asn_{106}-glycosylation modified recombinant chicken cystatins during 6 freezing-thawing cycles (i.e. freezing at -20°C for 14 h and thawing at 25°C for 10 h), there was still 93% of the original activity remained in the Asn_{106}-glycosylation

modified recombinant chicken cystatin, but only 65% and 63% remained in the non-modified recombinant and native cystatins, respectively (Table 1). This phenomenon suggested that the carbohydrate moiety on the Asn_{106}-glycosylation modified cystatin have a stabilizing effect on the cystatin.

Table 1. Stability of the native, recombinant and Asn_{106}-glycosylated recombinant chicken cystatin against freezing

Time (day)	0	1	2	3	4	5	6
Native cystatin activity[1] (units)	35.45	32.02	30.04	28.45	26.90	25.02	22.46
Relative residual activity (%)	100	90.32	84.73	80.23	75.87	70.56	63.35
Recombinant cystatin activity[2] (units)	147.23	138.52	132.76	127.60	101.75	100.73	95.36
Relative residual activity (%)	100	94.08	90.17	86.67	69.11	68.42	64.77
Glycosylated cystatin activity[3] (units)	156.32	153.02	153.45	150.36	150.76	146.35	146.01
Relative residual activity (%)	100	97.89	98.16	96.19	96.44	93.61	93.45

[1] The native chicken cystatin was purchased from Sigma Co Ltd., and purified again by Superose-12 chromatography.

[2,3] The recombinant chicken cystatin and its glycosylated form were expressed from pGAPZαC-cystatin transformed *Pichia pastoris* and purified by various steps.

Example 3-Thermal stability of recombinant and glycosylated recombinant chicken cystatin

The recombinant and glycosylated recombinant chicken cystatins were dissolved in 50 mM Tris-HCl buffer (pH 7.5) respectively, and then were placed at 30, 40, 50, 60, 70, 80, 90 or 100 °C for 30 min. Thereafter, the inhibitory activity of said recombinant and glycosylated recombinant chicken cystatin was measured. The result was shown in Figure 3, which indicated that the glycosylated recombinant chicken cystatin has a superior thermal stability to the non-glycosylated recombinant chicken cystatin in the range of 60 to 100 °C.

Example 4-Use of the recombinant and glycosylated recombinant chicken cystatin for the storage of surimi

Preparation of surimi

Fresh loin of mackerel was minced, to which 4x volume of a cold alkaline solution containing 0.4% of $NaHCO_3$, 4x volume of cold water and 2x volume of 0.3% aqueous HCl were sequentially added for water-bleaching, wherein each water-breaching took 5 min. Thereafter, the resulting product was subjected to centrifugal dewatering. After the fish tendon was removed, 3% sucrose solution, 3% sorbitol and 0.2% polyphosphate containing 50% sodium polyphosphate and 50% sodium pyrophosphate were added to the product, which in turn was homogenized by a kneader mixer and was stored in a refrigerator at -40 °C.

Effect of the recombinant and glycosylated recombinant chicken cystatins on the gel softening of fish meat jelly product

When mackerel surimi was kneaded in a 2.5% NaCl aqueous solution, a series of 0 to 12 active units of the recombinant or glycosylated recombinant chicken cystatin per 100 g of mackerel surimi were added (where the control did not contain any chicken cystatin), and the resulting products were kneaded homogeneously. The kneaded solutions were infused into casing materials with a diameter of 2.5 cm, and the products were then directly heated at 100 °C for 30 min., or were firstly put into a water bath of 50 °C for 2 hr and then were heated at 100 °C for 30 min. When the products were jelled, the jelled products were stored in a refrigerator at 4 °C for 12 hr. Thereafter, the strength of the jelled products was measured (wherein a 2.5 cm-high jelled sample was measured by an elastometer, which has a detection head with a diameter of 5 mm, at a detection velocity of 60 mm/min., and the strength (g x cm) of a jelled product was expressed by a product of breaking force (g) x deformation (mm)), and the jelled products were analyzed by electrophoresis (wherein 0.03 g of the jelled products was dissolved with heating at 50 °C in 2 ml of a buffer containing 2% SDS-8 M urea-2% β-Me-20 mM Tris-HCl, pH 8.0, and was analyzed by a non-continuous electrophoresis where the resolving gel was prepared by 10% acrylamide, the concentration of the stacking gel was 5%, the voltage for mini-gel electrophoresis was 100 V, and the resulting gel was stained by Coomassie brilliant blue G-250 and destained by 25% methanol and then dried). The results were shown in Table 2 and Figure 4. It was found that there was no significant difference in the strength of the jelled products to which were added more than 2

active units of the recombinant or glycosylated recombinant chicken cystatin.

Table 2. Breaking force (g) and deformation (mm) of mackerel surimi supplemented with the recombinant and glycosylated recombinant chicken cystatins expressed from *Pichia pastoris* X-33 strain*

	Recombinant cystatin		Glycosylated recombinant cystatin	
Inhibitory activity (units)**	Breaking force (g)	Deformation (mm)	Breaking force (g)	Deformation (mm)
0	277 ± 15.9 [a]	6.0 ± 0.31 [a]	277 ± 15.9 [a]	6.0 ± 0.31 [a]
1.0	355 ± 15.2 [b]	7.1 ± 0.36 [b]	344 ± 11.3 [b]	6.3 ± 0.20 [a]
2.0	432 ± 14.5 [c]	8.2 ± 0.42 [c]	380 ± 17.4 [c]	7.0 ± 0.24 [b]
4.0	480 ± 29.1 [cd]	8.9 ± 0.59 [c]	524 ± 22.9 [d]	8.6 ± 0.33 [c]
8.0	501 ± 27.5 [d]	9.1 ± 0.39 [c]	668 ± 28.4 [e]	10.2 ± 0.44 [d]
12.0	507 ± 17.2 [d]	9.0 ± 0.26 [c]	631 ± 37.1 [e]	10.0 ± 0.31 [d]

* Crude recombinant and glycosylated recombinant chicken cystatins obtained from culture broth of *Pichia pastoris* X-33 transformant was dialyzed against 20 mM phosphate buffer (pH 7.0).

** Values are the means of 10 determinations; values bearing unlike subscripts (a-e) in the same column are of significant difference ($p < 0.05$).

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